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Evaluation of the toxic effects of four anti-cancer drugs in plant bioassays and its potency for screening in the context of waste water reuse for irrigation

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HIGHLIGHTS

- Toxicological assays using seeds of *L. sativa* and *A. cepa* were carried out with CP, MTX, 5-FU and IM.
- The results indicated MTX as the most phytotoxic compound, followed by 5-FU, CP and IM.
- CP, MTX and 5-FU presented cytotoxic activity whereas CP, 5-FU and IM genotoxic potential.
- The four compounds showed significant formation of micronuclei.

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ABSTRACT

Anti-cancer drugs are compounds that are of high environmental relevance because of their lack of specific mode of action. They can be extremely harmful to living organisms even at low concentrations. The present study evaluated the toxic effects of four frequently used anti-cancer drugs against plant seedlings, namely Cyclophosphamide (CP), Methotrexate (MTX), 5-Fluorouracil (5-FU) and Imatinib (IM). The phytotoxicity experiments were performed with *Lactuca sativa* seedlings whereas cytotoxicity, genotoxicity and mutagenicity investigations were performed with the well-established *Allium cepa* assays. MTX was the most phytotoxic compound, followed by 5-FU, CP and IM. Significant differences in the Mitotic Indexes (MI) were observed in three of the studied compounds (MTX, 5-FU and CP), indicating potential cytotoxic activity of these substances. Chromosome aberrations were registered in cells that were exposed to 5-FU, CP and IM. All the four compounds caused the formation of micronucleated cells indicating mutagenic potential. Besides, the assays performed with MTX samples presented a high number of cell apoptosis (cell death). Although it is unlikely that the pharmaceuticals concentrations measured in the environment could cause lethal effects in plants, the obtained results indicate that these compounds may affect the growth and normal development of these plants. So, both tests can constitute important tools for a fast screening of environmental contamination e.g. in the context of the reuse of treated wastewater and biosolids of agricultural purpose.

1. Introduction

Nowadays around 4000 active pharmaceutical compounds, used as human and veterinary drugs, are available on the European market (Mompelat et al., 2009) among them about 100 anti-cancer drugs (Kümmerer et al., 2014). Due to an increased prevalence of cancer worldwide (Stewart and Wild, 2014), the consumption of anti-cancer drugs is expected to increase over the next years (Besse et al., 2012). Consequently their presence in the surface and wastewater is also expected to rise (Kümmerer et al., 2014; Zhang et al., 2013).

The first results confirming the presence of anti-cancer drugs in aquatic systems were published still in the 1980s (Aherne et al., 1985) followed by investigations in the 1990s (Aherne et al., 1990) and now in the 21st century (Besse et al., 2012).

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Nevertheless, there is still a lack of knowledge concerning the environmental fate of the drugs itself and its metabolites after excretion and possible risks connected to their presence in the aquatic environment (Kümmerer et al., 2014).

Although the measured low environmental concentrations compared to other groups of pharmaceuticals (Kosjek and Heath, 2011), the anti-cancer drugs require a special attention because they are mostly are non-selective in their modes of action, affecting both cancerous and non-cancerous cells and often causing severe systemic side effects (Allwood et al., 2002). Some of them interfere directly with the DNA and are recognized, even at low concentrations, as potentially fetotoxic, genotoxic, mutagenic and teratogenic substances in non-target organisms (Allwood et al., 2002).

Despite the benefits arising from the reuse of treated wastewater to irrigate agricultural crops and application of biosolids for agricultural purposes, some studies have demonstrated that plant uptake from water or soil spiked with pharmaceutical chemicals is associated with recycling of the waste products from WWTPs (Tanoue et al., 2012). Albeit the low measured concentrations of human pharmaceuticals in soils, due to the persistent nature of many pharmaceuticals and the repeated application of contaminated biosolids or irrigation water, there is a potential for accumulation to occur in soil systems (Schmidt and Redshaw, 2015). So, there is a growing concern that residual pharmaceutical chemicals have the potential to be taken up by edible plants and then enter the food supply (Tanoue et al., 2012).

Bioassays involving higher plants have been extensively used in environmental monitoring studies over the last years. Among them the assay with *Lactuca sativa* (lettuce) is one of the most common. Besides being quite simple and not expensive, this test has been accepted and validated for use in toxicity studies (Sobrero and Ronco, 2004) e.g. by the United States Environmental Protection Agency (U.S. EPA) and the Organization for Economic Co-operation and Development (OECD, 2003; U.S. EPA, 1996). Although *L. sativa* cannot be considered a representative species of the aquatic environment, the results generated in this assay can be used to evaluate the possible toxic effects of contaminants on plants that live near polluted water bodies (Sobrero and Ronco, 2004) or as a result of irrigation with reused water if contaminants are not fully removed in predicting treatment. Furthermore, it is a representative of main dicotyledon commercial crops and considered a standard species due to moderate sensitivity and high frequency of use in phytotoxicological tests (D'Abrosca et al., 2008; Hillis et al., 2011).

The use of assays with higher plants to evaluate the genotoxic effects of environmental pollutants has increased during the last years. According to Fiskesjö (1985) the facilities to store and handle plants, the good chromosomes conditions, the low costs and a good correlation with other systems are some reasons that make them very useful. Furthermore these tests have a great capacity to detect mutagenic effects in different environments and allow the evaluation of distinct genetic endpoints, which range from point mutations to chromosome aberrations (CA) in cells of different organs and tissues, such as leaves, roots and pollen (Leme and Marin-Morales, 2009).

Because of its high sensibility in detecting environmental chemicals and some structural advantages such as the large size and small number of their chromosomes ($2n = 16$), *Allium cepa* root-tip cells have been widely used in assays to evaluate chromosomes damages and disturbances in the mitotic cycle (Leme and Marin-Morales, 2009). Furthermore, the test involving this species is considered an easy handling assay and has advantages over other bioassays that require previous treatments of tested samples, as well as the addition of exogenous metabolic system, as in the Ames test. Besides all the advantages above mentioned, the *A. cepa* test has shown a good correlation when compared with other test systems, e.g. mammals and being even more sensitive than the Ames and the Microscreen test (Rank and Nielsen, 1994).

As already mentioned above, the bioassay with *A. cepa* allows the assessment of different endpoints. In this test, the screening of the cytotoxic potential is determined by the alterations of the Mitotic Index (MI), characterized by the total number of dividing cells in cell cycle. A significant reduction of the MI in comparison to the negative control may be due to a chemical action in the growth and development of exposed organisms. On the other hand, MIs higher than the negative control are results of an increase in cell division, which can be harmful to the cells, leading to a disordered cell proliferation and even to the formation of tumor tissues (Leme and Marin-Morales, 2009).

The evaluation of the chromosome aberrations (CA) has been used in the *A. cepa* test as a parameter to detect potentially genotoxic agents. Structural chromosomal alterations may be induced by several factors, such as DNA breaks, inhibition of DNA synthesis and replication of altered DNA. For the assessment of the potential genotoxic effects several types of CA found in the different phases of the cell cycle (prophase, metaphase, anaphase and telophase) are considered. Chromosome bridges and breaks, chromosome losses, fragments, delays, adherence, viscosity, multipolarity and C-metaphases are among the most common CA used to evaluate the genotoxicity in the *A. cepa* test.

Furthermore, the *A. cepa* test also enables the evaluation of the mutagenicity through the formation micronucleus (MN). For many authors, MN is the most effective and simplest endpoint to analyze the mutagenic effect promoted by chemicals. MN results from damages wrongly repaired (or not repaired) in the parental cells being easily measured in daughter cells as a structure similar to the main nucleus, but in a reduced size (Fenech, 2000). Therefore, MN arise from the development of some chromosome aberrations (CA), such as, chromosome breaks and losses or may still derive from other processes as polyploidization, in which they originate from the elimination of exceeding DNA of the main nucleus in an attempt to restore the normal conditions of ploidy (Fernandes et al., 2007; Leme and Marin-Morales, 2009).

Therefore, the present study aimed to assess the phytotoxicity, cytotoxicity, genotoxicity and mutagenicity of four anti-cancer drugs commonly used in anti-cancer treatments (Cyclophosphamide (CP), Methotrexate (MTX), 5-Fluorouracil (5-FU) and Imatinib (IM)) in two plant bioassays. Besides been among the most consumed anti-cancer drugs worldwide, the selected compounds are known not to be fully removed by biological and oxidative treatment (Besse et al., 2012; Booker et al., 2014; Steger-Hartmann et al., 1997; Yu et al., 2006).

2. Materials and methods

Since cytostatic drugs are potential cytotoxic, genotoxic, mutagenic and teratogenic compounds, their handling requires strict safety precautions (Allwood et al., 2002; Eitel et al., 1999). All stock solutions were prepared under a biological safety cabinet with laminar airflow. All the waste generated during the experiments was disposed and treated as hazardous and the instruments and glassware used were carefully cleaned before and after usage applying appropriate safety measures.

2.1. Test compounds, chemicals and seedlings

Cyclophosphamide monohydrate (CAS nr. 6055-19-2, 99%), methotrexate (CAS nr. 59-05-2, 98%) and imatinib mesylate (CAS nr. 220127-57-1, 99%) were kindly provided by Blausiegel Industry and Commerce Inc. (Cotia, SP, Brazil) whereas 5-fluorouracil (CAS nr. 51-21-8, 99%) was obtained from Sigma–Aldrich Biochemie